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Title
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Permalink
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Journal
Nature, 479(7371)

ISSN
0028-0836

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Publication Date
2011-10-01

DOI
10.1038/nature10501

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Nature. Author manuscript; available in PMC 2012 July 09.

Published in final edited form as:

Nature. ; 479(7371): 135–138. doi:10.1038/nature10501.

RNAi promotes heterochromatic silencing through replication-coupled release of RNA polII

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Abstract

Heterochromatin comprises tightly compacted repetitive regions of eukaryotic chromosomes. The inheritance of heterochromatin through mitosis requires RNA interference (RNAi), which guides histone modification [1] during the DNA replication phase of the cell cycle[2]. Here, we show that the alternating arrangement of origins of replication and non-coding RNA in pericentromeric heterochromatin results in competition between transcription and replication. Co-transcriptional RNAi releases RNA polymerase II (PolII), allowing completion of DNA replication by the leading strand DNA polymerase, and associated histone modifying enzymes[3] which spread heterochromatin with the replication fork. In the absence of RNAi, stalled forks are repaired by homologous recombination without histone modification.

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Author contributions. SC, DVI, AK, JR contributed equally to this work and are listed in alphabetical order. MZ, SC, DVI, AK, JR, FL, EdC, LM, AC and DG performed experiments, and SC analyzed the data. WZC, FA, BA and RM designed experiments and RM and MZ wrote the manuscript.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Information. Genomics data and analysis are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) accession number GSE30837. Individual cDNA sequences are available from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with accession numbers JN388396 to JN388565. Reprints and permissions information is available at www.nature.com/reprints.

The authors declare no competing financial interests.

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In fission yeast, the Rik1/CLRC (Recombination in K, Cryptic Locus Regulator) complex silences heterochromatin via Clr4 and Lid2, which methylate histone H3 lysine 9 (H3K9) and demethylate histone H3 lysine 4 (H3K4), respectively[2]. This complex is recruited in part by RNA interference, which processes non-coding transcripts found in the pericentromeric heterochromatin[1,4]. Interactions between the RITS (RNAi transcriptional silencing) complex and CLRC have recently been found[5,6], but spreading of the Rik1 complex into reporter genes depends on the catalytic activity of RNAi, and the mechanism remains unknown[7]. Recently, we found that Cdc20 and Mms19 interact with Rik1 and are required for histone modification[3]. Cdc20 is the catalytic subunit of the leading strand DNA polymerase Polε, while Mms19 is a regulatory subunit of the PolII transcription factor TFIIH. Both proteins participate in transcription coupled nucleotide excision repair (TC-NER) which depends on damage-stalled PolII to detect structural lesions in the DNA which are repaired by the Polε after PolII release[8].

The pericentromeric heterochromatin of fission yeast comprises outermost (*otr*) repeats called *dg* (5kb) and *dh* (1-6kb), flanked by innermost (*imr*) repeats (~6kb) containing clusters of tRNA genes (Fig. 1a). Histone H3 lysine-9 methylation is associated with *dg* and *dh* repeats (Fig. 1b), but ends abruptly at the tRNA clusters, and so is confined to heterochromatin[9]. The *dg* and *dh* repeats are transcribed by RNA polymerase II [10], and processed into siRNA clusters up to 4.5kb in length (Fig. 1b). To investigate the extent of siRNA precursor transcripts, we first cloned and sequenced *dh* and *dg* repeat complementary DNA from *dcr1*Δ mutants (Fig. 1c). Polyadenylation sites were then identified using RACE-PCR (Methods), and sequencing revealed they were located within the clusters of siRNA (Fig. 1c). In previous studies of *dcr1*Δ mutants, PolII enrichment was detected by ChIP[11], while transcriptional run-on (TRO) analysis indicated over-accumulation of forward (but not reverse) transcripts[1]. We found that these PolII ChIP (cen-*dg*) and TRO probes lie downstream of “forward” polyA sites (Fig. 1d), indicating inefficient termination and PolII readthrough in the absence of RNAi. To confirm readthrough, Northern blots of polyadenylated and total RNA from *dcr1*Δ, *ago1*Δ and *rdp1*Δ mutants were probed with strand specific probes. Transcripts corresponding to full length *dh* (1.3kb) and *dg* (1.3-2.3kb) cDNA clones were enriched in polyA+ RNA, as expected, but much longer readthrough transcripts up to 4.5kb could also be detected (Supplementary Fig. 1) indicating that polyadenylation was highly inefficient at these internal sites.

Inefficient polyadenylation is a strong indication of failure to release RNA polymerase II[12], and we hypothesized that slicing[7] and dicing[13] of nascent transcripts via RNAi promotes 3’-5’ degradation by the exosome[7] and release of RNA Polymerase II from the 3’ end[12]. The exosome is required for silencing consistent with this idea[1415]. To examine PolII release, we performed ChIP-seq with PolII antibodies, and found peaks of both poised (S5 phosphorylated) and elongating forms (S2 phosphorylated) of PolII in *dcr1*Δ mutants that corresponded to the polyadenylation sites on each strand (Fig. 1b,c). Peaks of siRNA accumulation mapped just downstream. Thus siRNA in WT cells accumulated where PolII was released (Fig. 1b).

siRNA accumulate during S phase[2] and we found that siRNA clusters ended abruptly at the replication origin homology regions contained within each repeat[16] (Fig. 1b). To assess the

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influence of DNA replication on PolII accumulation we blocked replication in high concentrations of hydroxyurea (HU) and performed ChIP-seq using PolII antibodies. In arrested *dcr1*Δ mutants, PolII accumulated throughout the *otr* repeats, but in dividing *dcr1*Δ cells, PolII accumulation was absent from replication origins (Fig. 2a and data not shown). To test if PolII was expelled by replication fork progression (Fig. 2b), HU-arrested *dcr1*Δ cells were released into the cell cycle (Fig. 2c). As predicted, accumulation at replication origins was quickly lost, and PolII was only found between origins, closer to promoters[10], in each subsequent S phase.

Failure to release RNA polymerase II during S phase is a strong and robust signal for DNA damage[8]. In order to monitor DNA repair, the HU-arrested cells contained a Rad22-fusion protein Rad22-YFP. Rad22 (Rad52 in budding yeast) is essential for homologous recombination (HR) and is associated with single stranded DNA ends early during DNA repair. Chromatin immunoprecipitation revealed that Rad22 ^{Rad52} was weakly associated with heterochromatic origins in wild-type cells arrested with HU, but quickly declined following release (Fig. 2c). In *dcr1*Δ mutants, on the other hand, Rad22 ^{Rad52} peaked early in each successive S phase, indicating engagement of the repair machinery during heterochromatin replication[17]. In order to exclude the impact of HU arrest on DNA damage, we also examined Rad22-YFP accumulation in untreated WT and *dcr1*Δ mutant cells by fluorescence microscopy (Supplementary Fig. 2). The results were consistent with chromatin IP, in that 6 times as many *dcr1*Δ than WT cells had Rad22 ^{Rad52} foci during septation (early S phase). Therefore, Dcr1 activity prevents DNA damage and the engagement of HR at the centromere.

We performed genetic tests to determine the role of RNAi in preventing DNA damage during S phase. DNA damage during replication can be rescued by HR repair, and we found that double mutants in the RecA homolog *rhp51* ^{rad51} and *dcr1*Δ or *ago1*Δ were inviable or formed microcolonies (Fig. 2d). A similar requirement for Rhp51 ^{Rad51} has been demonstrated for convergent stalled replication forks[18], which are protected from collapse in fission yeast by a stable replication-pausing complex comprising Swi1/Swi3 and Mrc1 (Mediator of replication checkpoint 1)[19]. Low concentrations of HU stalls replication forks, and we found that while *dcr1*Δ, *ago1*Δ and *rdp1*Δ cells were insensitive, double mutants with *swi3*Δ or *mrc1*Δ were very sensitive to low concentrations of HU (Supplementary Fig. 3). Similar results were obtained with Camptothecin (CPT) which causes arrest during S phase when the replication fork encounters the CPT-topoisomerase I complex. In genome-wide epistasis tests, mutants in more than 30 genes, mostly encoding proteins involved in DNA repair and histone modification interacted significantly with both *mrc1* and *dcr1*, forming a striking genetic network (Supplementary Table 1). This indicates that loss of Dcr1 activity engages replication fork protection.

In order to assess fork integrity, we examined replication of the repeats by 2D gel electrophoresis using probes from the *ura4* transgene, which was inserted into a passively replicated *dg* repeat on chromosome 1 (Fig. 2b). In WT cells, we detected strong X intermediates, indicative of joint molecules, as well as the expected fork or Y molecules (Fig. 3a). Similar X-DNA sister chromatid junctions arise at origins[20] but also at stalled replication forks[21]. These X-molecules were unaffected in *dcr1*Δ (Fig. 3b) but reduced in

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*mms19*Δ, in *swi6*Δ and especially in *clr4*Δ cells (Fig. 3c-e). Both Mms19 and Clr4 interact with Rik1, and Mms19 participates in transcription initiation[3]. Swi6 on the other hand is required to initiate replication within heterochromatic repeats[17], and recruitment depends on Clr4. Thus simultaneous replication and transcription of heterochromatic repeats promote local replication fork stalling.

In WT cells (Fig. 4a), modified histones recruit Swi6 and the Rik1 complex via chromo- and other domains. Swi6 promotes early replication, and the Rik1 complex interacts with DNA Polε, which allows spreading of histone modification along with fork progression[3]. Flanking tRNA genes (Fig. 1a) pause replication[22], preventing further spreading into neighboring euchromatin[9,23]. Transcription during S phase stalls the replication fork, accounting for interactions between the replication and transcription machineries[3], but RNAi releases PolII allowing replication to proceed. In the absence of RNAi (Fig. 4b), PolII remains stalled at replication forks and signals DNA repair by homologous recombination, which restarts blocked forks[24]. The Rik1 complex is lost along with the replisome, preventing spreading of heterochromatin into reporter genes, which lose H3K9 methylation entirely. Recombination also removes modified histones from at least one of the two daughter chromatids[25] reducing, but not eliminating, methylation of the repeats as previously observed[7].

We tested this model in several ways. First, we predicted that the interaction between the Rik1 complex and Polε should depend on RNAi, and we found that co-immunoprecipitation of Cdc20/ Polε with Dos2/Clr7 was reduced in *dcr1*Δ cells, along with H3K9me2 (Supplemental Fig. 4). Second, we observed that mutants in the cyclin-dependent PolII CTD kinase *Cdk9* display slow growth and loss of pericentromeric silencing and sRNA (Supplemental Fig. 5). *cdk9* is a central regulator of transcription elongation that links cell-cycle regulated pre-mRNA processing, co-transcriptional histone methylation and DNA damage[26]. Finally, Clr4 has recently been found to have additional roles in recruiting the RNA-induced transcriptional silencing (RITS) complex to accessory PolII factors[27], providing a potential mechanism for PolII release by RNAi. We found long transcripts indicative of strong transcriptional readthrough in *clr4*Δ mutant cells consistent with this model (Supplementary Fig. 1).

In the budding yeast *S. cerevisiae*, the Dicer-related RNAse III Rnt1 releases PolII during transcription termination[28] while in *E.coli*, failure of transcription termination stalls replication forks and triggers recombination[29], providing a precedent for the mechanism we propose. According to this mechanism, transcription during S phase triggers histone modification, so long as RNA polymerase is released by RNAi, and not by homologous recombination repair. In plants, fungi and invertebrates, heterochromatic silencing may involve similar mechanisms (Supplemental Table 1), while in mammals, both X inactivation and imprinting require transcription of non-coding RNA in dividing cells[30]. In each case, release of PolII during S phase, by RNAi or by other means, could allow fork restart and spreading of histone modification in a similar way.

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Methods summary

Non-coding transcripts were cloned from a cDNA phage library by hybridization to *dh* and *dg* consensus probes. Cloning and high throughput sequencing of sRNA was performed using the Illumina genome analyzer according to manufacturer's instructions. Two dimensional gel electrophoresis of replication intermediates from steady state cultures was performed with probes to the *otr1:ura4+* insertion. For ChIP experiments, cultures were arrested in 15mM HU for 4.5 hours, released and harvested at indicated times, to be crosslinked and processed for Chromatin immunoprecipitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Donna Roh and Tom Volpe for isolating cDNA clones. DI was supported by a NHMRC CJ Martin Postdoctoral Research Fellowship. MZ was supported by a fellowship from the Spanish Ministry of Science. This work was supported by grants BFU2008-01919 and Consolider-Ingenio CSD2007-00015 from the Spanish Ministry of Science and Innovation to FA, and NIH R01 GM076396 to ZC and RM.

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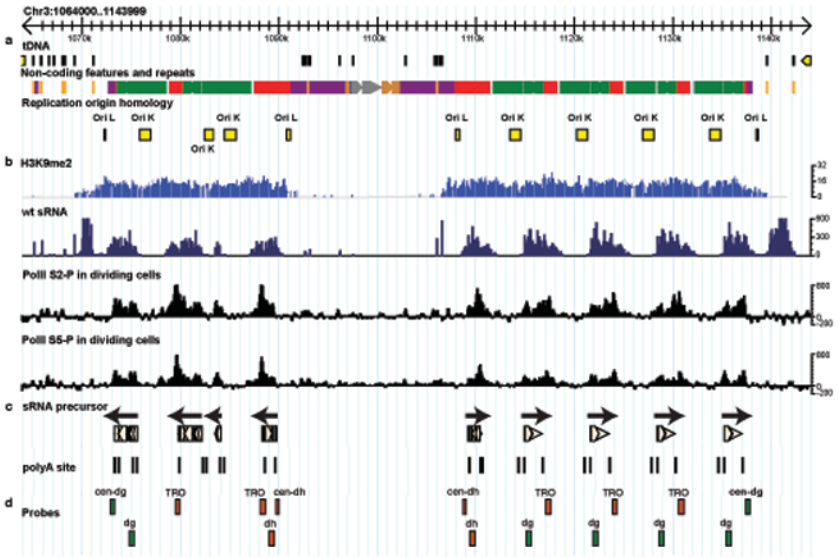

Figure 1. Transcription and replication of pericentromeric heterochromatin in fission yeast

a. Pericentromeric heterochromatin on Centromere 3. *dh* (red), *dg* (green) and imr (magenta) repeats are shown, bordered by tRNA genes (brown). Replication origins (yellow) are found in each repeat. **b**. Tiling microarrays of K9me2 ChIP (light blue) and clusters of small RNA sequences (dark blue) from wild-type cells. ChIP-seq reads corresponding to poised (S5-P) and elongating (S2-P) RNA polymerase II enriched in *dcr1*Δ cells relative to WT cells are in black. **c**. cDNA clones (beige) from *dcr1*Δ cells. PolyA sites are indicated as vertical lines and correspond to peaks of PolII. Arrows indicate the direction of “Forward” transcription. **d**. Alignment of probes used in previous studies indicates that regions enriched for PolII[11] (cen-*dg*) and transcriptional run-on probes[1] (TRO) lie downstream of forward orientation polyA sites

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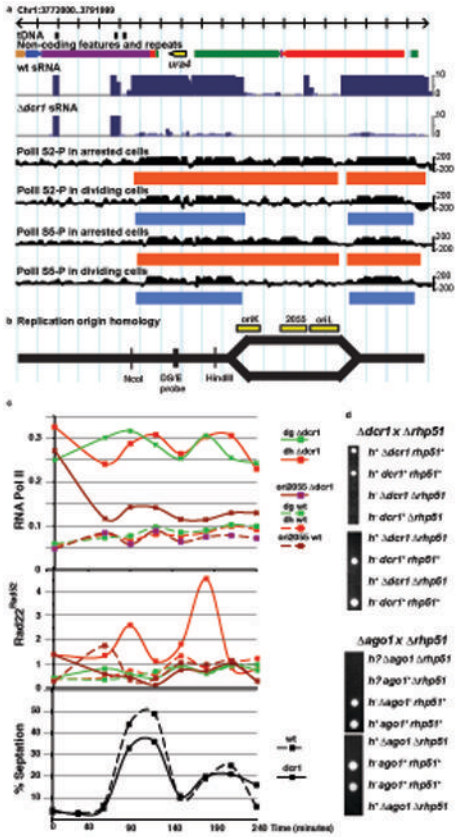

Figure 2. RNA interference and DNA replication restrict RNA polymerase II accumulation and prevent DNA damage

a. Small RNA (blue) and PolII ChIP-seq reads (black) and regions of significant enrichment (blue and red rectangles) from WT and *dcr1*Δ on the right arm of Centromere 1. **b**. A replication bubble is shown, initiated at one of the 3 origin homology regions at centromere 1 (yellow boxes). **c**. Chromatin immunoprecipitation for RNA PolII and Rad22^{Rad52} from HU-arrested and released wild-type (dashed lines) and *dcr1*Δ (solid lines). Cell cycle progression after release from HU block is monitored by septation index, which peaks coincident with S phase. **d**. Representative parental and non-parental di-type tetrads from crosses between *rhp51*Δ cells, defective in homologous recombination, and *dcr1*Δ or *ago1*Δ.

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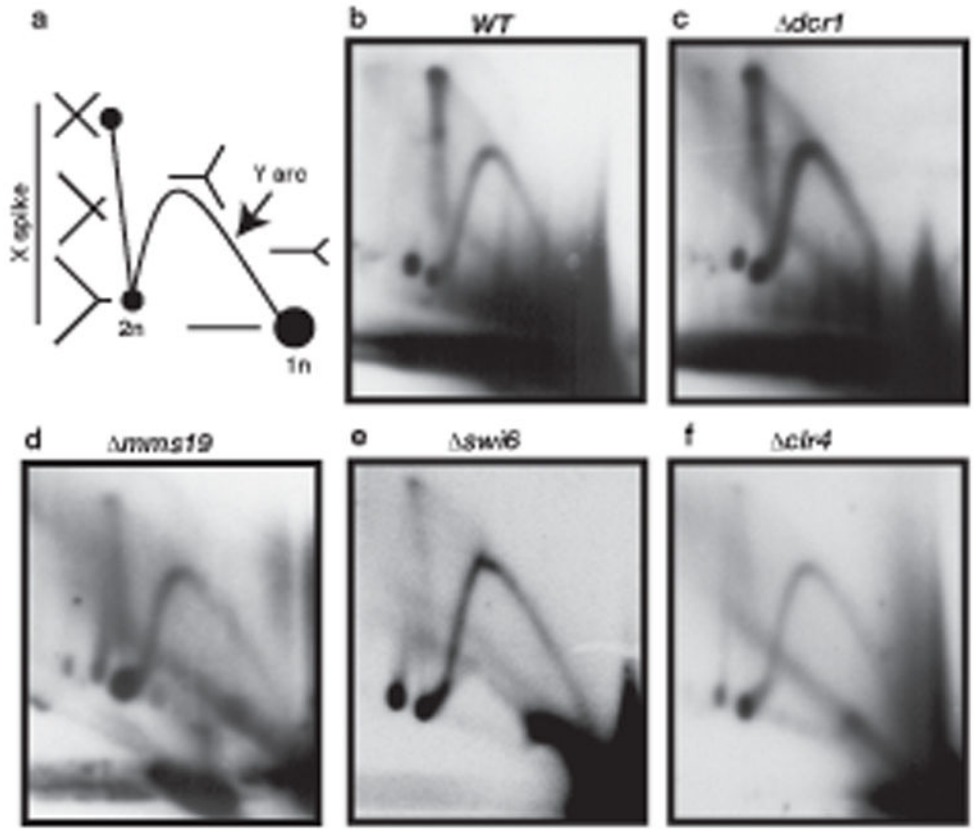

Figure 3. Replication fork stalling during heterochromatin replication

Replication intermediates in wild-type and mutant cells resolved by 2D gel electrophoresis and probed with the unique DS/E probe from the *ura4* transgene within the *dg* repeat on chromosome 1 (Fig. 2a). (**a**) A schematic of replication intermediates in 2D gels indicates joint molecules (X), and forks (Y). Junction molecules indicate fork stalling in (**b**) WT and (**c**) *dcr1*Δ mutant cells, and are reduced in (**d**) *mms19*Δ, (**e**) *swi6*Δ and (**f**) *clr4*Δ.

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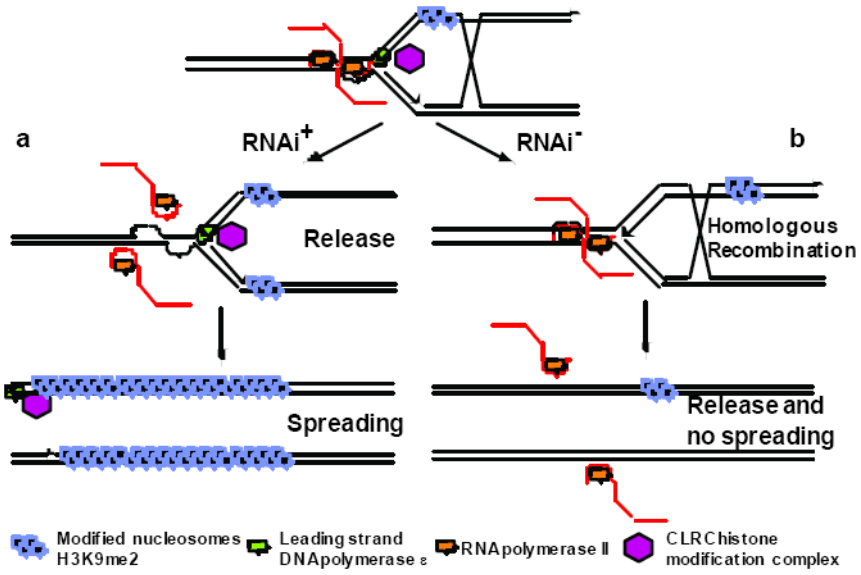

Figure 4. Replication-coupled transcriptional silencing through histone modification and RNAi

a. The Rik1 complex (red octagon) is recruited to heterochromatic replication forks by interactions with methylated histone H3K9me2 and with the leading strand DNA polymerase (Pol ε, green). Swi6 induces origin firing, but collision with RNA polymerase II (orange) stalls replication forks. RNAi releases PolII by processing of pre-siRNA transcripts (red lines) allowing leading strand DNA polymerase to complete DNA replication and the associated Rik1 histone modification complex (red hexagon) to spread histone modification (black circles).

b. In the absence of RNAi, origins fire but PolII is not released, stalling replication forks. Stalled PolII signals repair via homologous recombination instead. Recombination could in principle occur with sister chromatids (shown here) or with other copies of the same repeat (not shown). DNA polymerase ε and the associated Rik1 complex are lost along with the replisome, and fail to spread histone modification into neighboring reporter genes.